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Safety and tolerance of three probiotic strains in healthy infants: a multi-centre randomized, double-blind, placebo-controlled trial

S. Manzano[1,2#], J. De Andrés[1#], I. Castro[1], J.M. Rodríguez[1,2], E. Jiménez[1,2] and I. Espinosa-Martos[1,2*]

[1]Dpto. Nutrición, Bromatología y Tecnología de los Alimentos, Universidad Complutense de Madrid, Av. Puerta de hierro s/n, 28040 Madrid, Spain; [2]Probisearch S.L.U., C/ Santiago Grisolía, 2, 28760 Tres Cantos, Spain; [#]Both authors contributed equally; irene.espinosa@probisearch.com

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RESEARCH ARTICLE

Abstract

Some strains of species belonging to the genera *Bifidobacterium* and *Lactobacillus* are used in order to maintain health. Although these organisms have a long record of safe use, it is important to assess their safety and tolerance in potentially vulnerable populations, such as infants. The objective of this study was to evaluate the safety and tolerance of three probiotic strains (*Bifidobacterium longum* subsp. *infantis* R0033, *Bifidobacterium bifidum* R0071 and *Lactobacillus helveticus* R0052) in healthy infants aged 3 to 12 months. A multi-centre randomized, double-blind, placebo-controlled intervention study with 221 healthy full-term infants was conducted. Infants received either a placebo or one of the 3 probiotic strains (3×10^9 cfu) daily during an 8 week intervention period. Growth (weight, height and head circumference), adverse events (AEs)/serious adverse events (SAEs), concentrations of D-lactic acid in urine samples, characteristics of the stools and use of medication were collected for safety evaluation. All 4 groups were homogeneous with respect to age, gender, feeding type, ethnicity, height, weight and head circumference at the start of the study. The results showed that changes in growth (weight, height and head circumference) were equivalent in all 4 groups. No SAEs were reported. Total number of AEs recorded was equivalent in all groups. Thus, the use of *B. infantis* R0033, *L. helveticus* R0052 and *B. bifidum* R0071 in infancy is safe, and well tolerated.

Keywords: clinical trial, probiotics, healthy infants, safety and tolerance

1. Introduction

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) define probiotics as 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). The *Bifidobacterium* and *Lactobacillus* genera are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements (Frick *et al.,* 2007; Gourbeyre *et al.,* 2011; Macpherson and Harris, 2004). One of the mechanism by which *Bifidobacterium* and *Lactobacillus* are thought to inhibit the growth of pathogenic microorganisms is through the production of lactic, acetic, and other organic acids, with a consequent decrease of intraluminal pH. Moreover, *Bifidobacterium* and *Lactobacillus* strains may produce other antimicrobial compounds and compete with potentially pathogenic bacteria for nutrients and epithelial adhesion sites (Braegger *et al.,* 2011).

Probiotics are increasingly being administered to infants with the intention of improving health and, in fact, probiotic therapies are entering the therapeutic mainstream of paediatric diseases (Van Niel, 2005). The administration of probiotics as a single-species or as a multi-species product, in supplements or in follow-on infant formula given beyond early infancy may be associated with some health benefits. Studies have shown a reduction in the risk of non specific gastrointestinal infections, a reduced risk of antibiotic use, and a lower frequency of colic and/or irritability (Braegger *et al.,* 2011). Furthermore, it has been demonstrated that the use of certain *Lactobacillus* or *Bifidobacterium* probiotic strains reduces the risk of diarrhoea in infants and the incidence of respiratory infections and acute otitis media

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during the first year of life when compared with placebo (Chouraqui *et al.,* 2004; Rautava *et al.,* 2009; Szajewska *et al.,* 2001). Probiotics have also been demonstrated to be effective in the prevention of atopic dermatitis, reducing the risk by half compared with placebo (Kalliomäki *et al.,* 2001).

Probiotic blends have been administered to a large number of healthy infants in research studies without reported adverse events (AEs) but these studies were mostly efficacy studies looking at the different safety parameters only as a secondary outcome. In this context, studies in children (1 to 7 years old) with the combination of the probiotic strains *Bifidobacterium longum* subsp. *infantis* R0033, *Lactobacillus helveticus* R0052 and *Bifidobacterium bifidum* R0071 have found an improvement in their health compared with placebo (Cazzola *et al.*, 2010a,b; Mei and Chen, 2008). Recent cases of bacteraemia by *Lactobacillus* or *Bifidobacterium* in preterm, term infants and children have been reported (Bertelli *et al.*, 2015; Dani *et al.,* 2016; Thompson *et al.*, 2001). Although the patients with infections in these studies have had underlying conditions predisposing them to infection some species of lactobacilli can induce serious infections, including sepsis, pneumonia, and meningitis in compromised newborns and children (Dani *et al.*, 2016). Consequently, it is important to assess the safety and tolerance of single strains in a potentially more vulnerable population, such as infants (Braegger *et al.*, 2011).

Therefore, the aim of the present study was to evaluate the safety and tolerance of 3 probiotic strains (*B. longum* subsp. *infantis* R0033, *L. helveticus* R0052 and *B. bifidum* R0071) in healthy infants from 3 to 12 months in a randomised, double-blind, placebo-controlled study.

2. Materials and methods

Study design

A multi-centre randomised, double-blind, placebo-controlled, parallel-group, intervention study with 4 groups was conducted between June 2014 and April 2015 in health care centres from different areas of Madrid, Spain. Ethical committee approval was obtained from Hospital Universitario La Paz on 26 May 2014 (reference 4176). Written informed consent was obtained from the parents or legal guardians. The duration of the study was 12 weeks, divided into 3 different periods; 2 weeks run-in, 8 weeks treatment and 2 weeks follow-up (Figure 1).

During the study, 4 visits were conducted by the investigators/paediatricians: (1) at the start of the study (visit 1); (2) at the end of the run-in period (visit 2); (3) at the end of the treatment period (visit 3); and (4) at the end of the follow-up period (visit 4, Figure 1). The following data were collected at the study visits: type of feeding, growth parameters and events related to the health behaviour of the infant, non-scheduled visits resulting from a health problem, serious

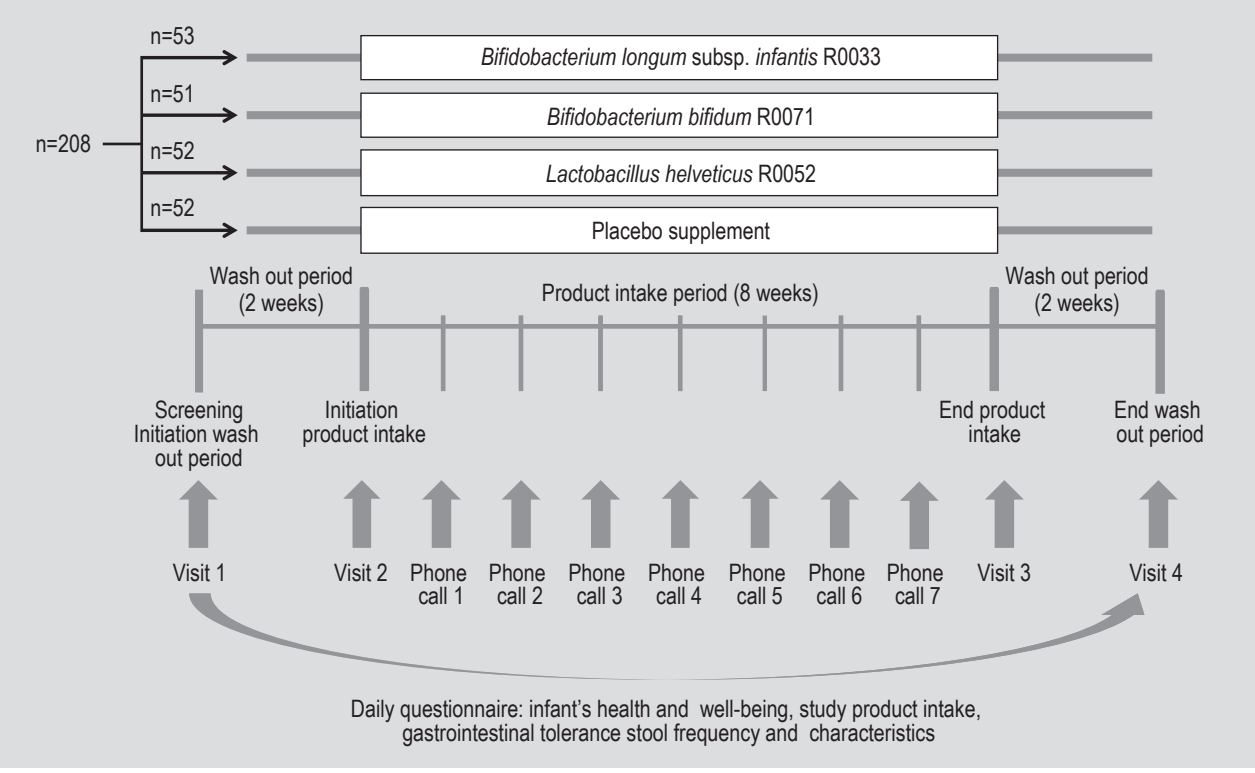

Figure 1. Schematic diagram of the study.

(S)AEs and concomitant medication. Additionally, weekly phone calls (n=7) were made during the treatment period in order to collect relevant data related to possible (S)AEs and to the infant's well-being (change in sleep behaviour, crying time, fever episodes, rashes, diarrhoea, unexpected visits to the doctor and antibiotic prescription).

Further, parents were instructed to complete a daily questionnaire to record the general health of the infant, day care attendance, type of feeding, introduction of new foods, vaccination and stools characteristics using the Amsterdam Stool Chart (Bekkali *et al.,* 2009). All the completed questionnaires and the unused study products were collected at visit 4. The study was conducted in accordance with the 'World Medical Association Declaration of Helsinki' (64th WMA General Assembly, Fortaleza, Brazil, October 2013).

Subjects

The recruited participants met the following inclusion criteria: healthy, full-term (≥37 weeks gestation at birth) and aged between 3 to 12 months old. The exclusion criteria were history of gastrointestinal (GI) disorders, GI surgery, metabolic disorders, immune deficiency, heart failure and/or cardiac medical history, surgery within one month prior to inclusion and antibiotic prescription one week before inclusion and during the wash out period. Investigators also excluded infants that had recently participated in other trials. Additionally, the use of probiotic supplements or probiotic infant formula during their participation in the study was not allowed.

The randomization was stratified for age and gender in order to obtain a homogeneous population for the 4 groups (*B. infantis* R0033; *B. bifidum* R0071; *L. helveticus* R0052 or placebo).

Study product

The investigational products were supplied by Lallemand Health Solutions (Montreal, Canada) as a fine white powder (1.5 g) packed in sealed sachets containing 3×10^9 cfu of either *B. infantis* R0033, *L. helveticus* R0052, *B. bifidum* R0071 (freeze-dried) and potato starch as the excipient. The placebo contained only the excipient and the appearance was the same as the probiotic products. Each participant received one sachet diluted in 10 ml water, breast milk or infant formula per day for a period of 8 weeks. Compliance with product intake was recorded by the parents in the daily questionnaire.

Study outcomes

The safety and tolerance outcomes of the study growth (weight, height and head circumference), (serious) adverse events ((S)AEs) and use of medication were assessed by the investigators/paediatricians during visits 1, 2, 3 and 4. Concentration of D-lactic acid was measured in urine samples collected during visits 1, 2, 3 and 4. The characteristics of the stools; frequency, quantity, consistency and colour were recorded daily by the parents according to the Amsterdam scale (Bekkali *et al.,* 2009). D-lactic acid amounts and stool characteristics were both also considered as safety and tolerance outcomes. Additionally, feeding option, childcare centre attendance and vaccination were also assessed. The enrolment questionnaire filled during visit 1 included following data: date of birth, age at inclusion, type of delivery, gender, ethnicity, feeding type and medical history.

AEs were defined as any untoward medical occurrence in an enrolled infant which does not necessarily have a causal relationship with the study product (FDA, 2016). All AEs were recorded and evaluated by the investigators/paediatricians for causality and severity. The AEs were assessed as SAEs if they were life-threatening, caused permanent harm, resulted in hospitalization or prolongation of existing hospitalization or resulted in persistent or significant disability or incapacity. All AEs were coded according to the Medical Dictionary for Regulatory Activities (MedDRA).

Concentration of D- lactic acid in urine samples

Urinary D-lactate concentration was determined using D-lactic acid/ L-lactic acid UV-method R-Biopharm kit (Darmstadt, Germany). Known amounts of D- and L-lactic acid were used as internal standard according the recommendations of manufacturer of the kit used.

Statistical methods for data analysis

Sample-size calculation was based on the primary outcome: infant growth during the study period. The objective was to show equivalence in weight gain of at least 6 g per day, a value that represented the minimum clinically relevant difference in weight (Koletzko *et al.,* 2009; Räihä *et al.,* 2002). Thus, the sample size estimated for assessing equivalence with a minimum weight gain of 6 g/day, with an alpha-level of 0.025 (two pairwise comparisons) and a statistical power of 80%, was 200 subjects (50 for each of the four groups). Assuming a dropout rate of approximately 10%, the total number of subjects to be included in the study was rounded off to 220. The sample-size calculation was performed with PASS 13, NCSS (Utah, USA).

In this study, an equivalence hypothesis was used for assessing growth, AEs, stool characteristics and other safety data recorded between the probiotic groups and the placebo. This means that the tested hypothesis was set forth to assess the following: H_0: $|\mu_{control} - \mu_{treated}| \geq \varepsilon$; H_1: $|\mu_{control} - \mu_{treated}| < \varepsilon$; thus when $P<0.05$, the H_0 was

rejected and groups were equivalent. According to the official growth tables of the Spanish Society for Paediatrics (AEP), the minimal clinical difference (ε) tolerated for growth are 6 g/day of weight gain, an increase of 0.05 cm/day in height and 0.018 cm/day for head circumference: so, these parameters were used to assess the equivalence between the different arms of the study. The other safety outcomes were analysed per week, so the results were expressed for each week of the study (Plaza-Díaz *et al.,* 2013; Wind *et al.,* 2010). Ordered-categorical variables and binary categorical data (yes/no) were analyzed by calculating the Z-statistic as described by Da Silva *et al.* (2008) and Mascha and Sessler (2011). For this purpose, the predetermined specified practical difference threshold of 1 was used for the ordered-categorical variables. Using different values for 'a biologically relevant statistical difference', Da Silva *et al.* (2008) established that 10% is the value that gives the best compromise between high statistical power and a low sample size, when equivalence testing for binary outcomes has to be evaluated. Therefore, the proportion of affirmative answers and a significant biological difference of 10% were employed for the analysis of the binary categorical data.

For the Intention To Treat (ITT) analysis, data from all randomly assigned infants who entered the study during the 12-week period was used. In contrast, the Per Protocol (PP) analysis excluded data from those subjects that during the study did not ingest the tested product for >3 consecutive days or 1 day per week, as stated in the protocol. The primary outcome was analysed in both the ITT and PP populations.

Prior to any other statistical analysis, general linear mixed models and post hoc ANOVA test of the model were used to assess any possible relationship of the confounding factors (age at inclusion and gender) with the arm of the study for all the outcomes. The same models were considered for different covariates (feeding type and new food introduction) with other outcomes (stool characteristics and possible relationships between fever and vaccines or unscheduled visits to the doctor). Only when a relevant relationship was obtained it was mentioned.

Statistical analyses were conducted using the Statgraphics Centurion XVII (version 17.0.16, Statpoint Technologies Inc., Warrenton, VA, USA) and R (version 3.0.2, R-project, http: www.R-project.org) software. Some statistics were directly calculated on MS Excel spreadsheets (Microsoft, Redmond, WA, USA), such as the z-statistic (Da Silva *et al.,* 2008; Mascha and Sessler, 2011).

3. Results

Study population

In this study, 221 infants were recruited and 202 completed the study (95 males and 103 females). Nineteen subjects withdrew, 13 of them before randomization (visit 2) and 6 during the treatment period (Figure 2). Four subjects did not comply with the product intake which meant a protocol deviation, and were therefore eliminated from the PP group. The results of the PP analysis are presented and include data collected during the treatment period of 198 infants.

Demographic characteristics of subjects recruited at the beginning of the study (visit 1) are shown in Table 1. No statistical differences in demographics were observed among the four arms of the study resulting in 4 homogeneous groups.

Growth parameters: weight, height and head circumference

Weight, height and head circumference were measured at all visits and the changes in anthropometric measures showed equivalence for each of the 3 treatment groups when compared to the placebo (Table 2).

Adverse events

No SAEs were recorded during the study. Recorded AEs of all participants who completed the study were classified according to the System Organ Class (SOC) of MedDRA and are shown in Table 3. A lack of power was observed when performing the statistical analysis. The z-statistic could only be calculated for the total number of AEs and the total number of participants with at least 1 AE per group. For the PP population significant equivalence to the placebo was observed in all 3 groups (Table 3). For the ITT population only the *B. infantis* R0033 group showed a total number of AEs not equivalent to that found in the placebo group (P=0.085). This non-equivalence was due to the high number of AEs (n=9) registered by one of the participants of the *B. infantis* R0033 group who was noncompliant with the product intake: 2 gastrointestinal; 4 respiratory, thoracic and mediastinal; 1 eye and 1 skin and subcutaneous tissue disorders; and 1 episode of fever, all these AEs were not related to product intake. Further, such non-equivalence was not detected for the number of participants with at least one AE ($P \leq 0.001$) or the number of 'possibly related' AEs (Table 3).

Gastro-intestinal symptoms, fever, rashes

Safety data were collected during study visits, phone calls and daily questionnaires. The frequencies of affirmative (yes) answers to questions regarding gastrointestinal symptoms

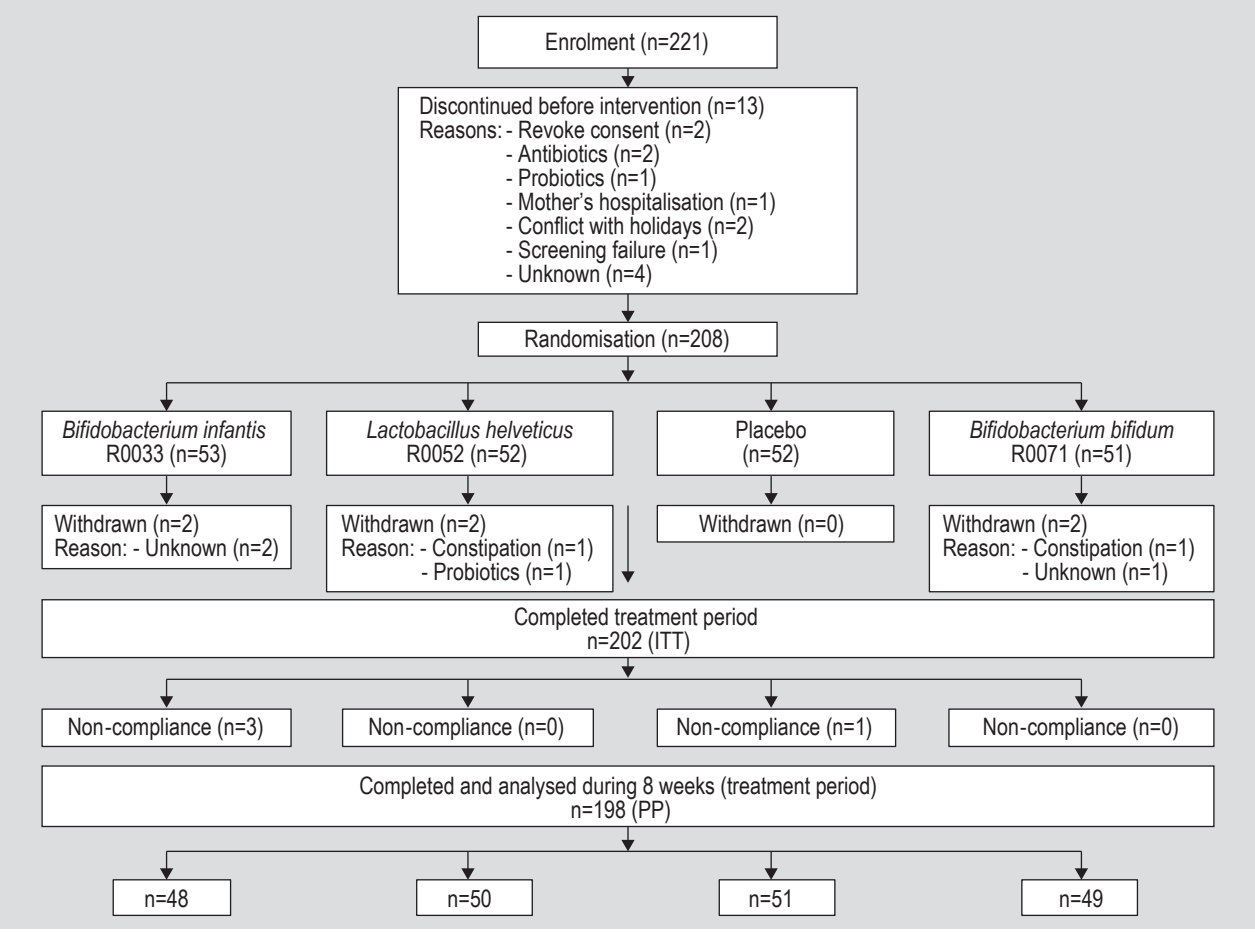

Figure 2. Flow chart of the study.

(diarrhoea), fever, rashes and unscheduled visits to doctor were registered weekly during the treatment period and at visits 3 and 4 for all the participants (Table 4). The results showed a low incidence in all of these safety variables for all 4 groups of the study, and were below the statistical biological difference of 10% (Da Silva *et al.,* 2008). Nevertheless, although the equivalence analysis could not be done, the behaviour of the treatment groups was similar to that observed for the control group. These results were also cross-examined with the daily questionnaires completed by the parents. No discordance was found between both sources of data (data not shown).

Changes in sleep and crying patterns

Changes in sleep and crying patterns, as general indicators of health status, were recorded in the daily questionnaires (Table 4). The incidence of these indicators was below the statistical biological difference of the 10% and was homogenous amongst all study groups (Da Silva *et al.,* 2008).

Concentration D-lactic acid in the urine samples

The concentration of D-lactic acid measured in urine samples from visits 1, 2, 3 and 4 were below the quantification limit of the method (33 μM) for all the tested samples (data not shown).

Stool characteristics

Daily characteristics of the stools were recorded by the parents according to the Amsterdam stool scale (Bekkali *et al.*, 2009). The statistical equivalence test of the weekly average records showed that the infants' stool characteristics in any of the three probiotic groups were equivalent to those of the placebo group (Table 5).

Use of medication

According to the data collected at visit 1, a low number of participants (6 infants) had concomitant medication and were randomly distributed in all four groups of the study. During the study period, 2 participants (1 in the *L. helveticus* R0052 group and the other in the *B. bifidum* R0071 group) used topical antibiotics.

Table 1. Demographic characteristics of the participants at the beginning of the study.[a]

	Bifidobacterium infantis R0033	*Lactobacillus helveticus* R0052	*Bifidobacterium bifidum* R0071	Placebo	*P*-value
Type of birth					0.478 [b]
Vaginal	40	38	36	42	
C-section	8	12	13	9	
Gender					0.859 [b]
Male	22	25	22	26	
Female	26	25	27	25	
Feeding type					0.720 [b]
Mother's milk	11	12	10	17	
Mixed	19	21	24	20	
Formula	18	17	15	14	
Ethnicity					0.383 [b]
Caucasian	48	49	49	51	
Others	0	1	0	0	
Age at inclusion (months)	6.00 (4.50-8.50)	6.50 (5.00-9.00)	7.00 (4.00-8.00)	6.00 (4.75-8.00)	0.948 [c]
Height (cm)	66.50 (62.25-71.00)	68.50 (64.25-71.00)	67.00 (65.00-71.00)	67.25 (63.87-70.00)	0.765 [c]
Weight (kg)	7.44±1.28	7.58±1.13	7.59±0.98	7.55±1.22	0.914 [d]
Head circumference (cm)	43.00 (41.60-44.75)	43.75 (42.00-45.00)	43.00 (42.00-44.00)	43.25 (41.50-44.12)	0.834 [c]

[a] Data are expressed as total frequencies for categorical outcomes; median (25Q-75Q) for quantitative outcomes not normally distributed and mean ± standard deviation when normally distributed.
[b] χ^2 test.
[c] Kruskal-Wallis test.
[d] ANOVA test.

Table 2. Evaluation of growth (height, weight and head circumference) of the participants during the intervention period.[a]

Treatment (56th day)	*Bifidobacterium infantis* R0033	*Lactobacillus helveticus* R0052	*Bifidobacterium bifidum* R0071	Placebo
Height (cm)	2.50 (2.00-3.40) [b]	2.60 (2.00-3.45) [b]	3.00 (2.00-3.80) [b]	3.00 (2.00-4.00)
Weight (kg)	0.79 (0.57-0.90) [b]	0.71 (0.49-0.90) [b]	0.63 (0.46-0.80) [b]	0.70 (0.56-0.98)
Head circumference (cm)	1.00 (1.00-1.85) [b]	1.00 (1.00-2.00) [b]	1.00 (1.00-2.00) [b]	1.00 (1.00-2.00)

[a] The change between visit 2 and 3 was expressed as median (25Q-75Q) for the recorded outcome.
[b] Significant equivalence with a 95% confidence level (P<0.05) when compared with the placebo group using a non-parametric tost-test.

4. Discussion

B. infantis R0033, *L. helveticus* R0052 and *B. bifidum* R0071 are strains with probiotic characteristics which have been shown to exert beneficial effects in animal models and in infants from 1 to 7 years of age (Cazzola *et al.*, 2010a,b; Mei *et al.*, 2008; Wine *et al.,* 2009). In the present study, the safety and tolerance of these probiotic strains was assessed in healthy infants aged 3 to 12 months over an intervention period of 8 weeks.

Growth is an essential outcome when evaluating the safety of probiotics as it is a sensitive, although nonspecific, sign of the overall health and nutritional status of an infant. Generally, growth studies should include measurements of, at least, weight, length and head circumference (Szajewska *et al.,* 2013). In this clinical study, the growth of all the participants was equivalent irrespective of the study product they consumed. These results are in agreement with those observed in other studies which reported that

Table 3. Adverse events for all participants that completed the study classified according to the System Organ Class (SOC) of the MedDRA.[a]

	Number of adverse events (AEs)				Number of participants with at least 1 AE				Number of 'Possibly-Related' AEs				Number of participants with at least 1 'Possibly-Related' AEs			
Body system	R0033 (n=48)	R0052 (n=50)	R0071 (n=49)	Placebo (n=51)	R0033 (n=48)	R0052 (n=50)	R0071 (n=49)	Placebo (n=51)	R0033 (n=48)	R0052 (n=50)	R0071 (n=49)	Placebo (n=51)	R0033 (n=48)	R0052 (n=50)	R0071 (n=49)	Placebo (n=51)
Gastrointestinal disorder	24	16	15	13	19	14	13	13	3	3	1	1	3	3	1	1
Infections and infestations	2	2	6	5	2	2	6	3	0	0	0	0	0	0	0	0
Bone and joint injuries	0	4	1	4	0	4	1	4	0	0	0	0	0	0	0	0
Nervous system disorders	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
Respiratory, thoracic and mediastinal disorders	19	14	10	9	13	9	7	7	0	1	0	0	0	1	0	0
Skin and subcutaneous tissue disorders	0	2	0	2	0	2	0	2	0	0	0	0	0	0	0	0
Eye disorders	4	2	2	2	2	2	2	2	0	0	0	0	0	0	0	0
Ear and labyrinth disorders	3	3	3	1	3	2	3	1	0	0	0	0	0	0	0	0
Fever (LLT)	7	7	2	5	6	5	2	5	0	0	0	0	0	0	0	0
Total	57[b]	50[b]	39[b]	42	45[b]	40[b]	34[b]	38	3	4	1	1	3	4	1	1

[a] R0033 = *Bifidobacterium longum* subsp. *infantis* R0033; R0052 = *Lactobacillus helveticus* R0052; R0071 = *Bifidobacterium bifidum* R0071; LLT = low level term.

[b] Significant equivalence with a 95% confidence level (P<0.05).

Table 4. Absolute and relative frequencies of affirmative (yes) answers to the questions asked at the weekly phone calls and visits 3 and 4.[1]

	Absolute frequencies (relative frequencies)			
	Bifidobacterium infantis **R0033 (n=459)**	***Lactobacillus helveticus*** **R0052 (n=450)**	***Bifidobacterium bifidum*** **R0071 (n=441)**	**Placebo (n=468)**
Fever	14 (0.032)	20 (0.044)	13 (0.029)	12 (0.027)
Rash	5 (0.012)	6 (0.013)	5 (0.011)	3 (0.007)
Diarrhoea	22 (0.051)	13 (0.029)	13 (0.029)	14 (0.032)
Unscheduled visit to doctor	32 (0.074)	31 (0.069)	21 (0.048)	25 (0.057)
Change in sleeping habits	47 (0.109)	61 (0.036)	41 (0.088)	39 (0.088)
Crying	53 (0.123)	80 (0.178)	67 (0.143)	59 (0.134)

[1] Questions asked at the weekly phone calls and at visit 3 and 4: Fever: Did your infant suffer a fever episode in the last week?; Rash: Did your infant have a rash on any part of his/her body in the last week?; Diarrhoea: Did your infant have diarrhoea in the last week?; Unscheduled visit to doctor: Did your infant have an unscheduled visit to the doctor last week? If yes, why?; Change in sleeping habits: Were there any changes in your infant's sleeping habits?; Crying: Did your infant cry excessively in the last week?

the consumption of probiotics did not affect growth (Lee *et al.*, 2015).

Adverse events, concentration of D-lactic acid in urine samples and tolerance parameters such as characteristics of the stools were evaluated in this study. The AEs were classified according to the MedDRA SOC classification (Räihä *et al.,* 2002). None of the participants suffered a SAE during the study and the number of AEs recorded during the entire duration of the study was under the

Table 5. Average of affirmative (yes) answers to questionnaires per week recorded for stools' characteristics (number, quantity, consistency and colour) and equivalence test.[1]

		Bifidobacterium infantis R0033 Median (IQR)	*Lactobacillus helveticus* R0052 Median (IQR)	*Bifidobacterium bifidum* R0071 Median (IQR)	Placebo Median (IQR)
Week 3	N stools[2]	2.00 (1.28-2.43)[a]	2.00 (1.57-2.57) [a]	1.86 (1.43-2.57) [a]	1.71 (1.14-2.86)
	Quantity[3]	3 (2-3)[a]	3 (2-3) [a]	3 (2-3.5) [a]	3 (2-3)
	Consistency[4]	3 (2-3)[a]	3 (2-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour[5]	2 (1-2)[a]	1 (1-2) [a]	2 (1-2) [a]	2 (1-2)
Week 4	N stools	1.71 (1.28-2.43) [a]	2.00 (1.32-2.71) [a]	1.57 (1.14-2.36) [a]	1.71 (1.14-2.75)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (2-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	2 (1-2) [a]	2 (1-2)
Week 5	N stools	1.86 (1.28-2.57) [a]	2.00 (1.18-2.53) [a]	1.86 (1.07-2.57) [a]	1.77 (1.25-2.75)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-4) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (2-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 6	N stools	1.85 (1.28-2.36) [a]	2.21 (1.28-2.57) [a]	1.85 (1.14-2.43) [a]	1.78 (1.14-2.46)
	Quantity	3 (3-3) [a]	3 (2-3) [a]	3 (3-3.5) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (2-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 7	N stools	1.71 (1.21-2.43) [a]	2.07 (1.28-2.57) [a]	1.71 (1.28-2.07) [a]	1.71 (1.26-2.61)
	Quantity	3 (3-3) [a]	3 (2.25-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (2.25-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 8	N stools	1.86 (1.21-2.50) [a]	2.00 (1.17-2.53) [a]	1.71 (1.14-2.14) [a]	1.78 (1.14-2.71)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-4) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (2-3) [a]	3 (2-3) [a]	3 (2-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 9	N stools	1.67 (1.21-2.36) [a]	1.86 (1.28-2.57) [a]	1.71 (1.00-2.17) [a]	2.00 (1.25-2.43)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (2-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (2.75-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 10	N stools	1.86 (1.28-2.43) [a]	2.00 (1.28-2.57) [a]	1.71 (1.14-2.15) [a]	1.93 (1.28-2.57)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (2.5-3) [a]	3 (3-3) [a]	3 (2-3) [a]	3 (3-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 11	N stools	1.86 (1.14-2.28) [a]	1.93 (1.41-2.43) [a]	1.71 (1.21-2.15) [a]	1.86 (1.28-2.43)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (3-3) [a]	3 (2.25-3) [a]	3 (2.50-3) [a]	3 (3-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)
Week 12	N stools	1.84 (1.15-2.32) [a]	1.86 (1.30-2.43) [a]	1.67 (1.17-2.36) [a]	1.86 (1.15-2.71)
	Quantity	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Consistency	3 (3-3) [a]	3 (3-3) [a]	3 (3-3) [a]	3 (3-3)
	Ordered coded colour	1 (1-2) [a]	1 (1-2) [a]	1 (1-2) [a]	1 (1-2)

[1] Values with a superscript letter have a significant equivalence with a 95% confidence level ($P<0.05$) when compared with the placebo group using a non-parametric tost-test. The statistically significant difference was established at 1.

[2] N Stools: the average number of stools per week (mean) was calculated and rounded to an integer.

[3] Quantity: the most frequent quantity of stools per week (mode) is shown with a range of: 1 – Smear; 2 – less than 25%; 3 – between 25 and 50%; 4 – more than 50%.

[4] Consistency: the most frequent consistency of stools per week (mode) is shown with a range of: 1 – watery; 2 – soft; 3 – formed; 4 – hard.

[5] Ordered colour: first, the most frequent colour of stools per week (mode) with a range of I, II, III, IV, V and VI was taken into account. Then, this outcome was ordered according to the total proportions observed for each one of the categories, following the same criteria (high to low proportions) as described by Bekkali *et al.* (2009).

minimum relevant difference of 10%. The total number of AEs recorded was equivalent between the 4 groups and, therefore, they could not be attributed to the study product administered to the infants. These results are in accordance with those observed in other studies, further supporting the safety of certain lactobacilli and bifidobacteria strains and promoting their use as probiotics (Allen *et al.,* 2010; Chouraqui *et al.,* 2008; Dekker *et al.,* 2009; Weizman and Alsheikh, 2006).

To date no case of D-lactic acidosis because of intake of D-lactate-producing *Lactobacillus* has been documented in healthy infants. Reviewing the literature, all of the D-lactic acidosis cases were identified in infants with short bowel syndrome. Conversely, it has been shown that administration of a D-lactate-producing *Lactobacillus* species to children with short bowel syndrome, leads to a faster remission of the symptomatology than with probiotic strains that produce only L-lactate (Connolly and Lönnerdal, 2004). In this work, the levels of D-lactate in the urine samples analysed were below the quantification limit of the method, 33 µM or 3.6 – 105 mmol/mol of creatinine, considering reference ranges of creatinine in infants less than 2 years of life (http://tinyurl.com/7el5m6r, 2015). The urinary D-lactate concentrations reported previously in healthy infants after consumption of different probiotic strains indicated variability in D-lactate concentration that was unlikely to be attributed to *Lactobacillus* intake (Haschke-Becher *et al.* 2000, 2008; Papagaroufalis *et al.*, 2014). This fact highlights the lack of relation between the intake of these strains and D-lactate amounts in urine.

Finally, stool characteristics may help in the assessment of tolerance of a probiotic (Saavedra *et al.*, 2004). In this study, stool characteristics were evaluated weekly and compared with the placebo group. All of the three probiotic strains were found equivalent to the placebo showing that the consumption of these probiotic strains was well tolerated by infants aged between 3 to 12 months. In conclusion, the use of *B. longum* subsp. *infantis* R0033, *L. helveticus* R0052 and *B. bifidum* R0071 in infancy is safe and well tolerated.

Conflict of interest

This work was supported by Lallemand Health Solutions Inc. (Montreal, Canada). The authors declared no conflict of interest.

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